

#6

**Docket No. GC636-2**

(A). Western blot analysis of hIL-3 protein variants produced by *B. subtilis* 168 transformed with plasmid pLATIL3 (lane 1), pLATIL3BStag (expression of hIL-3 with a C-terminal *B. subtilis* SsrA tag (AA-tag): hIL-3-AGKTN<sup>S</sup>FNQNVALAA (SEQ ID NO:1); lane 2), pLATIL3DDtag (expression of hIL-3 with a DD-tag: hIL-3-AGKTN<sup>S</sup>FNQNVALDD (SEQ ID NO:2); lane 3), and pLATIL3ECTag (expression of hIL-

3 with a C-terminal *E. coli* SsrA tag (EC-tag): hIL-3-AANDENYALAA (SEQ ID NO:3); lane 4). Culture supernatants of cells entering the stationary phase were collected and analyzed by SDS-PAGE and Western blotting with anti-hIL-3 antibody.

On page 7, please replace the paragraph starting on line 12 with the following:

**Figure 7.** A native protein and examples of the types of tags encompassed by the instant invention. (A) Depicts the sequence of human interleukin-3 (SEQ ID NO:25); the (native) signal peptide is in bold. (B) Depicts the IL-3 sequence encoded by plasmid pLATIL3 (SEQ ID NO:26). The sequence of AmyL[ss]-interleukin-3 is for hIL-3 secretion in *Bacillus*: the AmyL signal peptide is in bold, and this expressed IL-3 lacks the last four amino acids (LAIF) of native hIL-3. The tag is in italics. (C) Depicts IL-3 with a tag that is a substitution of the native protein's terminal two amino acids (SEQ ID NO:27). The tag is in italics. (D) Depicts a tag that is an addition to the native protein's carboxy terminus (SEQ ID NO:28). Here the sequence of hIL3 as encoded by pLATIL3 with the SsrA-DD tag at the C-terminus [hIL3-DD] (signal peptide in **bold**, C-terminal tag in *italics*) is shown.

On page 12, please replace the paragraph starting on line 13 with the following:

In another preferred embodiment, the tag may be a modified *Bacillus* SsrA tag. In an especially preferred embodiment the modified tag has the sequence AGKTNSFNQNVALDD (SEQ ID NO:2) or AGKTNSFNQNVALKK (SEQ ID NO:4).

On page 20, please replace the paragraph starting on line 3 with the following:

*B. subtilis* SsrA has been isolated and sequenced several years ago (Ushida et al. 1994 tRNA-like structures in 10Sa RNAs of *Mycoplasma capricolum* and *Bacillus subtilis*. Nucleic Acids Res. 22:3392-3396) and the sequence of the proteolysis tag encoded by *B. subtilis* SsrA ((A)GKTNSFNQNVALAA SEQ ID NO:1) has been predicted (Williams 2000. The tmRNA website. Nucleic Acids Res. 27:165-166). Recently, Wiegert and Schumann (2001. SsrA-mediated tagging in *Bacillus subtilis*. J. Bacteriol. 183:3885-3889) showed that the ClpXP protease is responsible for the degradation of intracellular SsrA-tagged proteins in *B. subtilis*. The instant invention

provides for an enhanced protein stability via enhance protease resistance. In particular, the extracellular protease CtpA, and perhaps one or more of the major extracellular proteases of *B. subtilis*, play a role in the degradation of an extracellular, heterologous protein that was tagged by the SsrA system. It is a benefit that tagged proteins according to the present invention are more resistant to proteolysis.

On page 22, please replace the paragraph starting on line 12 with the following:

**Possible tags that may be used**

Tags may either be added to the carboxy terminus of a protein or substituted for the amino acids of the protein's carboxy terminus. If the protein has been tagged by the addition of amino acid residues the tag is preferably up to 20 additional residues preferably about 15, more preferred 1-14, even more preferred 1-11, and most preferred 1-3, wherein the last one or two amino acid residues are charged. Figure 7D depicts a protein with a tag added on to its carboxy terminus (SEQ ID NO:28). In this depiction the tag is 14 amino acid residues long.

On page 22, please replace the paragraph starting on line 21 with the following:

In the alternative, the tag may replace between 1 and 5 amino acids in the protein's carboxy terminus. In the substituted tag the amino acids are charged. In a preferred embodiment the last 5 amino acids are replaced with the tag. In another preferred embodiment the last 4 amino acids are replaced with the tag. In yet another preferred embodiment the last 3 amino acids are replaced with the tag. In a more preferred embodiment the last amino acid is replaced with the tag. In a most preferred embodiment the last 2 amino acids are replaced with the tag. Figure 7C depicts a substitution tagged protein (SEQ ID NO:27). In this depiction the final two amino acid residues of the native protein have been replaced with two charged amino acid residues.

On page 23, please replace the paragraph starting on line 22 with the following:

To study the degradation of SsrA-tagged proteins in *B. subtilis*, variants of pLATIL3 were made in which h-IL3 is expressed with different short peptide tags added to the COOH-terminus of h-IL3:

Variant 1: plasmid pLATIL3-BStag; expresses the hIL3 variant hIL3-AA: hIL3 with an apolar C-terminal *B. subtilis* SsrA-tag [GKTNSFNQNVALAA] (SEQ ID NO:5)

Variant 2: plasmid pLATIL3-DDtag; expresses the hIL3 variant hIL3-DD: hIL3 with a negatively charged C-terminal tag [GKTNSFNQNVALDD] (SEQ ID NO:6), this variant differs from variant 1 (hIL3-AA) only in the last two C-terminal amino acids (two aspartic acids (DD) instead of two alanine (AA) residues)

Variant 3: plasmid pLATIL3-ECtag; expresses the hIL3 variant hIL3-ECAA: hIL3 with an apolar C-terminal *E. coli* SsrA tag [AANDENYALAA] (SEQ ID NO:3)

On page 24, please replace the paragraph starting on line 29 with the following:

Transformation of competent cells was used to transfer DNA (plasmids, linear DNA) into *B. subtilis* (Harwood et al. 1990). PCR (polymerase chain reaction) was carried out with High Fidelity Platinum Taq DNA Polymerase (Life technologies) and if required PCR fragments were purified with the Qiaquick PCR purification kit (Qiagen). DNA primers were from Life technologies and DNA sequencing was performed by BaseClear (Leiden, The Netherlands). Plasmid pLATIL3TERM was obtained by PCR on pLATIL3 with the primers pLATIL3SXHfw (5' GTC GAC CTC GAG ACC CCA AGC TTG GCG TAA TC 3') (SEQ ID NO:7) and pLATIL3T3rv (5' GTC GAC CTC GAG CGG CAG AAT CTT TTT TTG ATT CTG CCG CAA AGT CGT CTG TTG AGC CTG 3') (SEQ ID NO:8). The resulting DNA fragment was purified, digested with XhoI, self-ligated, and transformed directly into *B. subtilis*. One of the plasmid clones, found to be correct by DNA sequencing, was designated pLATIL3TERM. This plasmid holds the transcription terminator of the *folC* gene (present in primer pLATIL3T3rv) at the 3' end of

the AmyL-hIL-3 gene, just in front of an in-frame stop codon. Plasmid pLATILBStag was obtained by a PCR on pLATIL3 with the primers pLATIL3T2FW (5' CTG CAG CTC GAG GAT ATC GTC GAC CGG CAG AAT CAA AAA AAG ATT CTG CCG ACC CCA AGC TTG GCG TAA TC 3') (SEQ ID NO:9) and pL3BStagRV (5' CTT CTA CTC GAG TCA GGC AGC TAA TGC TAC GTT TTG GTT AAA ACT GTT AGT TTT GCC TGC GCT CAA AGT CGT CTG TTG AGC 3') (SEQ ID NO:10). The resulting PCR fragment was purified, digested with XhoI, self-ligated, and transformed into *B. subtilis*. A few clones were checked by DNA sequencing and one correct clone was selected and named pLATIL3BStag. Plasmid pLATIL3DDtag and pLATIL3Ectag were made in the same way but instead of primer pL3BStagRV, primer pL3DDtagRV (5' CTT CTA CTC GAG TCA GTC GTC TAA TGC TAC GTT TTG GTT AAA ACT GTT AGT TTT GCC TGC GCT CAA AGT CGT CTG TTG AGC 3') (SEQ ID NO:11) and primer pL3EctagRV (5' CTT CTA CTC GAG TCA AGC TGC TAA AGC GTA GTT TTC GTC GTT TGC TGC GCT CAA AGT CGT CTG TTG AGC 3') (SEQ ID NO:12) were used, respectively. To construct *B. subtilis*  $\Delta$ ssrA mutants, ssrA and its flanking regions (approximately 2.2 kb) was amplified by PCR with the primers pSsrAFW (5' CAG CTC CGT CTG AGG AAA AAG 3') (SEQ ID NO:13) and pSsrARV (5' CGA AGT GGG CGA TTT CTT CCG 3') (SEQ ID NO:14) and cloned into pCR2.1-TOPO, resulting in plasmid pTPSsrA. Plasmid pSsrASp was obtained by inserting a pDG1726-derived Sp resistance marker (Guérout-Fluery et al. 1995. Antibiotic-resistance cassettes for *Bacillus subtilis*. Gene 167:335-336) into the unique SacI site in the ssrA gene of pTPSsrA. Finally, *B. subtilis* 168  $\Delta$ ssrA and WB600  $\Delta$ ssrA were obtained by a double cross-over recombination event between the disrupted ssrA gene of pSsrASp and the chromosomal ssrA gene in *B. subtilis* 168 and WB600, respectively. SsrA<sup>DD</sup> expressing *B. subtilis* strains were made as follows: a fragment consisting of a 5' end part of ssrA including the ssrA promoter region, was amplified with the primers pSsrAHindIIIfw (5' TTC TAA AAG CTT AGT GCT TGA TTC GAA AAT CAG GCC TGT G 3') (SEQ ID NO:15) and pSsrADDintRV (5' GAG CTC GCT GCG CTT ATT AGT CGT CTA ATG CTA CGT TTT GGT TAA 3') (SEQ ID NO:16); contains the alteration of the two alanine codons in the SsrA tag sequence into codons for aspartic acid residues). In addition, an overlapping 3' end part of ssrA was amplified with the primers pSSrADDintFW (5' TTA ACC AAA ACG TAG CAT TAG ACG

ACT AAT AAG CGC AGC GAG CTC 3') (SEQ ID NO:17); also containing the alteration of the two alanine codons into codons for two aspartic acid residues) and pSsrASphIRV (5' CCT CCG TGC ATG CTT CCT CTT ATT TAT TGA CAG AAA TCT G 3') (SEQ ID NO:18). Both fragments were assembled in a fusion PCR with primers pSsrAHindIII<sub>FW</sub> and pSsrASphIRV, and cloned in pCR2.1-TOPO, resulting in plasmid pSsrADD. The correct sequence of the fusion product in pSsrADD was confirmed by DNA sequencing. Next, a selective marker (the Tc resistance cassette derived from pDG1515; Guérout-Fluery et al. 1995. Antibiotic-resistance cassettes for *Bacillus subtilis*. Gene 167:335-336) that functions in *B. subtilis*, was cloned into the EcoRV site of pSsrADD, resulting in plasmid pSsrADD<sub>Tc</sub>. Finally, *B. subtilis* 168 *IssrA*<sup>DD</sup> and WB600 *IssrA*<sup>DD</sup> were obtained by a Campbell-type integration (single cross-over) of pSsrADD<sub>Tc</sub> into one of the disrupted *ssrA* regions on the chromosome of *B. subtilis* 168  $\Delta$ *ssrA* and WB600  $\Delta$ *ssrA*, respectively. These strains contain an active copy of the *ssrA*<sup>DD</sup> gene on the chromosome (under control of the native *ssrA* promoter) and a disrupted copy of wild-type *ssrA* (insertion of the Sp resistance marker), as confirmed by PCR. To construct *B. subtilis* WB600  $\Delta$ *ctpA*, WB600 was transformed with chromosomal DNA of BSE-23. In BSE-23, the *ctpA* gene is replaced by a spectinomycin resistance cassette (Edwin Lee, Genencor International Palo Alto, unpublished). WB600  $\Delta$ *yvjB* was obtained as follows: *yvjB* and its flanking regions (approximately 3.5 kb) was amplified by PCR with the primers pYvjBFW (5' AGA GTT TTA AAT CTC TCG GGA GAA ACA CAT GGA TGA CAT T 3') (SEQ ID NO:19) and pYvjBRV (5' TGT ATA TGT AAA TTT CAG ATC ATC ATA AAT ATC TGC TAT T 3') (SEQ ID NO:20) and cloned in pCR2.1-TOPO, resulting in plasmid pTPYvjB. Plasmid pTPYvjB<sub>Tc</sub> was obtained by replacing an internal SmaI-AccI fragment of the *yvjB* gene in pTPYvjB with a pDG1515-derived Tc resistance marker (Guérout-Fluery et al. 1995. Antibiotic-resistance cassettes for *Bacillus subtilis*. Gene 167:335-336). Finally, *B. subtilis* WB600  $\Delta$ *yvjB* was obtained by a double cross-over recombination event between the disrupted *yvjB* gene of pTPYvjB<sub>Tc</sub> and the chromosomal *yvjB* gene. To construct *B. subtilis* WB600 *lclpP*, the 5' end region of the *clpP* gene was amplified by PCR with the primers pClpPEcoFW (5' CTT ACC GAA TTC GTG AAG GAG GAG CAT TAT G 3') (SEQ ID NO:21) containing a EcoRI site, and pClpPBamRV (5' GCC TTT GGA TCC GGC TGC AAG CAG GAA CGC 3') (SEQ ID

NO:22) containing a BamHI site. The amplified fragment was cleaved with EcoRI and BamHI, and cloned in the corresponding sites of pMutin2 (Vagner et al. 1998. A vector for systematic gene inactivation in *Bacillus subtilis*. Microbiology 144:3097-3104), resulting in plasmid pMutClpP. *B. subtilis* WB600 lclpP was obtained by a Campbell-type integration (single cross-over) of pMutClpP into the clpP region on the chromosome. Cells of this strain are depleted for ClpP by growing them in medium without IPTG (Vagner et al. 1998).

On pages 28-30, please replace Table 1 with the following:

Table 1. Plasmids and Strains

Plasmid/Strain	Properties	Reference
pLATIL3	derivative of pGB/IL-322: contains the human <i>IL-3</i> gene fused to the sequence encoding the signal peptide of <i>B. licheniformis</i> $\alpha$ -amylase ( <i>amyL-hIL-3</i> ); the <i>amyL-hIL-3</i> gene fusion is under control of the amylase promoter; 4.3 kb; Nm <sup>R</sup>	Van Leen et al. 1991. Production of human interleukin-3 using industrial microorganisms. Biotechnology 9:47-52.
pLATIL3TERM	derivative of pLATIL3; contains the transcription terminator of the <i>B. subtilis</i> <i>folC</i> gene inserted just in front of the stop codon of <i>amyL-hIL-3</i> ; 4.1 kb; Nm <sup>R</sup>	This work
pLATIL3BStag	derivative of pLATIL3; contains <i>amyL-hIL-3</i> fused at the 3' end to the sequence encoding the <i>B. subtilis</i> SsrA peptide tag (AGKTNSFNQNALAA SEQ ID NO:1); 4.2 kb; Nm <sup>R</sup>	This work
pLATIL3DDtag	derivative of pLATIL3; contains <i>amyL-hIL-3</i> fused at the 3' end to the sequence encoding a variant SsrA-DD-tag (AGKTNSFNQNALDD SEQ ID NO:2); 4.2 kb; Nm <sup>R</sup>	This work
pLATIL3ECTag	derivative of pLATIL3; contains <i>amyL-hIL-3</i> fused at the 3' end to the sequence encoding the <i>E. coli</i> SsrA peptide tag (AANDENYALAA SEQ ID NO:3); 4.2 kb; Nm <sup>R</sup>	This work
pCR2.1-TOPO	TA cloning vector for PCR products; 3.9 kb; Ap <sup>R</sup> , Km <sup>R</sup>	Invitrogen
pTPSSrA	pCR2.1-TOPO derivative; carrying the <i>ssrA</i> gene + flanking regions; 6.1	This work

Plasmid/Strain	Properties	Reference
	kb; Ap <sup>R</sup> ; Km <sup>R</sup>	
pSsrASp	derivative of pTPSsrA for the disruption of <i>ssrA</i> ; 7.0 kb; Ap <sup>R</sup> ; Km <sup>R</sup> ; Sp <sup>R</sup>	This work
pSsrADD	pCR2.1-TOPO derivative; carrying a <i>ssrA</i> <sup>DD</sup> gene variant: the last two codons of the tag sequence in <i>ssrA</i> (gct gcc) encoding two alanines are changed into gac gac, encoding two aspartic acid residues; 4.6 kb; Ap <sup>R</sup> ; Km <sup>R</sup>	This work
pSsrADDTc	derivative of pSsrADD; carrying <i>ssrA</i> <sup>DD</sup> and a Tc resistance cassette; for integration of <i>ssrA</i> <sup>DD</sup> on the <i>B. subtilis</i> chromosome; 6.8 kb; Ap <sup>R</sup> ; Km <sup>R</sup> ; Tc <sup>R</sup>	This work
pTPYvjB	pCR2.1-TOPO derivative; carrying the <i>yvjB</i> gene + flanking regions; 7.4 kb; Ap <sup>R</sup> ; Km <sup>R</sup>	This work
pTPYvjBTc	derivative of pTPYvjB for the disruption of <i>yvjB</i> ; 8.9 kb; Ap <sup>R</sup> ; Km <sup>R</sup> ; Tc <sup>R</sup>	This work
pMutin2	pBR322-based integration vector for <i>B. subtilis</i> ; containing a multiple cloning site downstream of the <i>Pspac</i> promoter, and a promoterless 1998 <i>lacZ</i> gene preceded by the RBS of the <i>spoVG</i> gene; 8.6 kb; Ap <sup>R</sup> ; Em <sup>R</sup>	Vagner et al. 1998. A vector for systematic gene inactivation in <i>Bacillus subtilis</i> . Microbiology <b>144</b> :3097-3104.
pMutClpP	pMutin2 derivative; carrying the 5' part of the <i>B. subtilis clpP</i> gene; 8.9 kb; Ap <sup>R</sup> ; Em <sup>R</sup>	This work
<b>Strains</b>		
<i>E. coli</i>		
TOP10	<i>F mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 deoR araD139 Δ(ara-leu)7697 galU galK rpsL (Str<sup>R</sup>) endA1 nupG</i>	Invitrogen
XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [FφproAB lac<sup>R</sup>ZDM15 Tn10 (Tet<sup>r</sup>)]</i>	Stratagene
<i>B. subtilis</i>		
168	<i>trpC2</i>	Kunst et al. 1997. The complete genome sequence of the Gram-positive bacterium <i>Bacillus subtilis</i> . Nature <b>390</b> :249-256.



Plasmid/Strain	Properties	Reference
168 $\Delta$ ssrA	<i>trpC2</i> , <i>ssrA</i> ; Sp <sup>R</sup>	This work
168 <i>IssrA</i> <sup>DD</sup>	<i>trpC2</i> , <i>IssrA</i> <sup>DD</sup> ; Tc <sup>R</sup> ; integration of pSsrADDTc in <i>ssrA::spec</i> in 168 $\Delta$ ssrA	This work
WB600	<i>trpC</i> , <i>nprE</i> , <i>aprE</i> , <i>epr</i> , <i>bpf</i> , <i>mpr</i> , <i>nprB</i>	Wu et al. 1991. Engineering a <i>Bacillus subtilis</i> expression-secretion system with a strain deficient in six extracellular proteases. J. Bacteriol. <b>173</b> :4952-4958.
BSE-23	<i>ctpA</i> ; Sp <sup>R</sup>	E. Lee, unpublished
WB600 $\Delta$ ctpA	<i>trpC</i> , <i>nprE</i> , <i>aprE</i> , <i>epr</i> , <i>bpf</i> , <i>mpr</i> , <i>nprB</i> , <i>ctpA</i> ; Sp <sup>R</sup>	This work
WB600 $\Delta$ yvjB	<i>trpC</i> , <i>nprE</i> , <i>aprE</i> , <i>epr</i> , <i>bpf</i> , <i>mpr</i> , <i>nprB</i> <i>yvjB</i> ; Tc <sup>R</sup>	This work
WB600 <i>lclpP</i>	<i>trpC</i> , <i>nprE</i> , <i>aprE</i> , <i>epr</i> , <i>bpf</i> , <i>mpr</i> , <i>nprB</i> , <i>Pspac-clpP</i> ; <i>clpP-lacZ</i> ; Em <sup>R</sup>	This work
WB600 $\Delta$ ssrA	<i>trpC</i> , <i>nprE</i> , <i>aprE</i> , <i>epr</i> , <i>bpf</i> , <i>mpr</i> , <i>nprB</i> , <i>ssrA</i> ; Sp <sup>R</sup>	This work
WB600 <i>IssrA</i> <sup>DD</sup>	<i>trpC</i> , <i>nprE</i> , <i>aprE</i> , <i>epr</i> , <i>bpf</i> , <i>mpr</i> , <i>nprB</i> , <i>IssrA</i> <sup>DD</sup> ; Tc <sup>R</sup>	This work

On page 32, please replace the paragraph starting on line 19 with the following:

RNA isolation and Northern blotting. RNA was isolated with the TRIzol method according to the protocol provided by the manufacturer (Life technologies), but with one modification: cells were incubated for 10 min at 37 °C with lysozyme (2 mg/ml) prior to lysis in TRIzol solution. Northern blotting was performed after electrophoresis of RNA through gels containing formaldehyde (Sambrook et al. 1989). To this purpose, Hybond-N+ nylon membrane from Amersham Pharmacia Biotech was used. The SsrA-specific probe was amplified by PCR with the primers SsrAFRWDP (5' ACG TTA CGG ATT CGA CAG GGA TGG 3') (SEQ ID NO:23) and SsrAREVP (5' GAG TCG AAC CCA CGT CCA GAA A 3') (SEQ ID NO:24). Labeling of the probe, hybridization and detection was performed with the ECL direct nucleic acid labeling and detection system from Amersham Pharmacia Biotech according to the manufacturer's instructions.

On page 33, please replace the paragraph starting on line 25 with the following:

Western blot analysis. To obtain anti-BsSsrA-tag antibodies (antibodies that recognize proteins with a C-terminal *B. subtilis* SsrA-tag), synthetic peptide AGKTNSFNQNVALAA (SEQ ID NO:1) (coupled via an amino-terminal cysteine residue to KLH carrier) was injected into rabbits (Eurogentec). Serum of the final bleed of one of the rabbits was selected for affinity purification, and this purified serum was used in the Western blot procedures. Antibodies against human IL-3 were mouse monoclonals (Van Leen et al. 1991. Production of human interleukin-3 using industrial microorganisms. Biotechnology 9:47-52). Immunoblotting and detection was performed with alkaline phosphatase-labeled conjugate and the BM Chromogenic Western Blotting kit (Roche Diagnostics) according to the instructions of the manufacturer.

On page 34, please replace the paragraph starting on line 8 with the following:

**Stability of hIL-3 variants with different C-terminal tags produced by *B. subtilis*.**

To further investigate whether the *B. subtilis* SsrA tag functions as a degradation signal for secreted proteins, three variants of plasmid pLATIL3 were created. Plasmid pLATIL3BStag contains a gene variant encoding hIL-3 fused at the C-terminus to the *B. subtilis* SsrA peptide tag (AGKTNSFNQNVALAA SEQ ID NO:1), plasmid pLATIL3ECtag contains a gene variant encoding h-IL3 fused at the C-terminus to the *E. coli* SsrA tag (AANDENYALAA SEQ ID NO:3). The third plasmid pLATIL3DDtag contains a gene encoding h-IL3 fused at the C-terminus to the sequence encoding a DD-tag (AGKTNSFNQNVALDD SEQ ID NO:2). This tag is equal to the *B. subtilis* SsrA-tag (AA-tag), but instead of two alanines at the extreme C-terminus it contains two aspartic acid residues. The DD-tag was suspected to be relatively resistant to proteolytic degradation, as observed for *E. coli* (Abo et al. 2000. SsrA-mediated tagging and proteolysis of LacI and its role in the regulation of *lac* operon. EMBO J. 19:3762-3769; Roche et al. 1999). The extracellular proteins produced by cells of *B. subtilis* 168 containing pLATIL3, pLATIL3BStag, pLATIL3DDtag, or pLATIL3ECtag, were analyzed by Western blotting (Fig. 3A). The amount of the hIL-3-DDtag present in the medium was found to be roughly 5 times higher than that of wild-type hIL-3, hIL-3-AAtag or hIL-3-ECtag. Human interleukin-3 molecules produced by wild-type *B. subtilis* are relatively unstable due to proteolytic degradation, and the results represented in figure 3A suggest that addition of

a C-terminal SsrA-tag does not lead to increased degradation of hIL-3 molecules. It is important to note, however, that in *E. coli* proteins tagged cotranslationally by the SsrA system are degraded more rapidly than proteins with essentially the same sequence in which the SsrA tag is DNA encoded (Gottesman et al. 1998. The ClpXP and ClpAP proteases degrade proteins with carboxy-terminal peptide tails added by the SsrA-tagging system. Genes Dev. 12:1338-1347). The results obtained with pLATIL3TERM (Fig. 2) indicate that this is also true for *B. subtilis*. Strikingly, addition of the DD-tag (with two charged, polar residues at the extreme C-terminus) leads to a higher level of extracellular hIL-3, indicating that DD-tagged hIL-3 is less susceptible to proteolytic degradation. To explore this further, a pulse-chase assay was performed with the *B. subtilis* strain 168 (pLATIL3BStag) and 168 (pLATIL3DDtag) (Fig. 3B and 3C). The initial level (chase time = 0 min) of hIL-3-DDtag in the medium is approximately 4 times higher than that of hIL-3-AAtag. In addition, the hIL-3-AAtag variant was degraded with a half-life of < 2 min, whereas the half-life of hIL-3-DDtag was somewhat increased (approximately 5 min). The latter observation supports that DD-tagged hIL-3 is less susceptible to extracellular proteases compared to hIL-3 with an AA-tag. However, the observation that the initial level of hIL3-DDtag in the medium is considerably higher than that of hIL3-AAtag indicates that hIL3-AAtag is also subject to proteolytic degradation before the molecules reach the medium, e.g. during passage of the cell wall of *B. subtilis*.

On page 35, please replace the paragraph starting on line 25 with the following:

**Detection of cotranslationally SsrA-tagged hIL-3 secreted by WB600 and by cells expressing an SsrA<sup>DD</sup> variant.** To detect SsrA-tagged h-IL3 molecules secreted by *B. subtilis* and to identify proteases that have a role in the degradation of SsrA-tagged hIL-3, two different approaches were used. First, pLATIL3TERM was expressed in WB600, a *B. subtilis* strain lacking six extracellular proteases (Wu et al. 1991. Engineering a *Bacillus subtilis* expression-secretion system with a strain deficient in six extracellular proteases. J. Bacteriol. 173:4952-4958), which may be responsible for the degradation of extracellular, SsrA-tagged hIL-3. In the medium of a culture of WB600 (pLATIL3TERM), a band was detected reacting with antibodies against hIL-3 as well as

with antibodies raised against the predicted *B. subtilis* SsrA-tag (Fig. 4, lane 3 and 8). This band is absent in the medium of *B. subtilis* 168 (pLATIL3TERM) (lanes 2 and 7) and WB600  $\Delta$ ssrA (pLATIL3TERM) (lanes 4 and 9). Thus, hIL-3 molecules translated from mRNAs that lack termination codons are tagged by *B. subtilis* SsrA. The fact that these tagged molecules react with antibodies raised against the predicted *B. subtilis* SsrA peptide tag (AGKTNSFNQNVALAA SEQ ID NO:1) indicates that this prediction, which was based on comparative sequence analysis of SsrA sequences of several bacteria (Williams 2000), was correct. In addition, it can be concluded that at least one of the major extracellular proteases of *B. subtilis* (those that are absent in WB600) plays a role in the degradation of extracellular, SsrA-tagged h-IL3. When SsrA is absent, stalled ribosomes are released by an SsrA-independent mechanism, referred to as 'run-off translation' (Williams et al. 1999. Resuming translation on tmRNA: a unique mode of determining a reading frame. EMBO J. 18:5423-5433). The upper band in lane 4 probably represents the run-off translation product of full-length hIL-3 mRNA from pLATIL3TERM, while the bands with lower molecular weight are most likely degradations products thereof. It seems that some run-off translation product is also formed when SsrA is present (lane 3), but it cannot be excluded that this band is just an N-terminal degradation product of SsrA-tagged hIL-3.

On page 36, please replace the paragraph starting on line 27 with the following:

As a second approach to detect SsrA-tagged proteins, we constructed *B. subtilis* strains that express an SsrA variant (SsrA<sup>DD</sup>), in which the final two codons of the peptide reading frame are changed to encode aspartic acid residues instead of alanines. As mentioned above, it was shown in *E. coli* that an SsrA<sup>DD</sup> variant mediates the addition of a peptide tag that does not lead to rapid degradation (Abo et al. 2000; Karzai et al. 1999. SmpB, a unique RNA-binding protein essential for the peptide-tagging activity of SsrA (tmRNA). EMBO J. 18:3793-3799). Evaluation of the antibodies that were raised against the predicted *B. subtilis* SsrA tag (AGKTNSFNQNVALAA SEQ ID NO:1) showed that they recognize the hIL-3 fused at the C-terminus to either the wild-type tag (AA-tag) or the protease resistant DD-tag (AGKTNSFNQNVALDD SEQ ID NO:2) (data not shown). Human IL-3 molecules tagged by SsrA<sup>DD</sup> and subsequently

secreted (Fig 4, lanes 5 and 10) are indeed relatively more stable than hIL-3 molecules tagged by wild-type SsrA (lanes 3 and 8), even in the six-fold protease negative strain WB600. The level of full-length SsrA<sup>DD</sup>-tagged hIL-3 in the medium is somewhat higher than that of (wild-type) SsrA-tagged hIL-3 (Fig. 3, compare lane 10 with lane 8) and relatively few degradation products of SsrA<sup>DD</sup>-tagged hIL-3 were detected with anti-hIL-3 antibody (compare lane 5 with lane 3). This observation suggests that besides the major extracellular proteases that are deleted in WB600, one (or more) additional protease is involved in the degradation of SsrA-tagged hIL-3. Therefore, we studied the role of three other proteases with respect to degradation of SsrA-tagged hIL-3.

In the claims: Please replace claims 37, 38, and 48 with the following:

37. The recombinant protein of interest of Claim 34 wherein said positively charged residue is R.
38. The recombinant protein of interest of Claim 29 wherein said tag is selected from the group comprising SsrA<sup>RR</sup> (SEQ ID NO:30), SsrA<sup>DD</sup> (SEQ ID NO:2), SsrA<sup>KK</sup> (SEQ ID NO:4), and SsrA<sup>EE</sup> (SEQ ID NO:29).
48. The nucleic acid molecule of claim 46 wherein the charged amino acid residue is R.

**REMARKS**

Entry of the above amendment prior to examination is respectfully requested.

Attached hereto is a marked-up version of the changes made to the specification and claims. The attached pages are captioned "**Version with Markings to Show Changes Made.**"

**I. Amendments**

The specification has been amended in accordance with 37 C.F.R. §1.821 through 1.825 to add the Sequence Listing.

The specification has been amended in accordance with 37 C.F.R. §1.821(d) to add SEQ ID NO:s.

Claim 38 has been amended in accordance with 37 C.F.R. §1.821(d) to add SEQ ID NO:s. The claim has further been amended to correct the peptide tag from SsrA<sup>NN</sup> to SsrA<sup>RR</sup>. Claims 37 and 48 have been amended to correct a typographical error. Support for the amendment to claim 38 can be found on page 12, lines 3-6 where the tag is described as preferably having at least one charged amino acid residue, and on page 10, lines 27-28 where the amino acid represented by R is described as positively charged. Support for claims 37 and 48 can be found throughout the specification as filed. For example, see page 10, lines 17-22. Additionally, one of skill in the art would recognize the error as the amino acid asparagine is not charged.

No new matter is introduced by way of these amendments.

Accordingly, in view of the above remarks, it is submitted that this application is now ready for allowance. Early notice to this effect is solicited.



The Commissioner is hereby authorized to charge the fees necessitated by the filing of these documents, or to charge any additional fees under 37 C.F.R. 1.16 and 1.17, or to credit any overpayment, to Deposit Account No. 07-1048.

If in the opinion of the Examiner a telephone conference would expedite the prosecution of the subject application, the Examiner is encouraged to call the undersigned at (650) 846-7615.

Respectfully submitted,

Date: January 18, 2002

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### Version with Markings to Show Changes Made

#### In the specification:

#### **Figure 3.** Stability of hIL-3 variants with different C-terminal tags.

(A). Western blot analysis of hIL-3 protein variants produced by *B. subtilis* 168 transformed with plasmid pLATIL3 (lane 1), pLATIL3BStag (expression of hIL-3 with a C-terminal *B. subtilis* SsrA tag (AA-tag): hIL-3-AGKTNSFNQNVALAA (SEQ ID NO:1); lane 2), pLATIL3DDtag (expression of hIL-3 with a DD-tag: hIL-3-AGKTNSFNQNVALDD (SEQ ID NO:2); lane 3), and pLATIL3ECTag (expression of hIL-3 with a C-terminal *E. coli* SsrA tag (EC-tag): hIL-3-AANDENYALAA (SEQ ID NO:3); lane 4). Culture supernatants of cells entering the stationary phase were collected and analyzed by SDS-PAGE and Western blotting with anti-hIL-3 antibody.

**Figure 7.** A native protein and examples of the types of tags encompassed by the instant invention. (A) Depicts the sequence of human interleukin-3 (SEQ ID NO:25); the (native) signal peptide is in bold. (B) Depicts the IL-3 sequence encoded by plasmid pLATIL3 (SEQ ID NO:26). The sequence of AmyL[ss]-interleukin-3 is for hIL-3 secretion in Bacillus: the AmyL signal peptide is in bold, and this expressed IL-3 lacks the last four amino acids (LAIF) of native hIL-3. The tag is in italics. (C) Depicts IL-3 with a tag that is a substitution of the native protein's terminal two amino acids (SEQ ID NO:27). The tag is in italics. (D) Depicts a tag that is an addition to the native protein's carboxy terminus (SEQ ID NO:28). Here the sequence of hIL3 as encoded by pLATIL3 with the SsrA-DD tag at the C-terminus [hIL3-DD] (signal peptide in **bold**, C-terminal tag in *italics*) is shown.

In another preferred embodiment, the tag may be a modified Bacillus SsrA tag. In an especially preferred embodiment the modified tag has the sequence AGKTNSFNQNVALDD (SEQ ID NO:2) or AGKTNSFNQNVALKK (SEQ ID NO:4).

*B. subtilis* SsrA has been isolated and sequenced several years ago (Ushida et al. 1994 tRNA-like structures in 10Sa RNAs of *Mycoplasma capricolum* and *Bacillus subtilis*. Nucleic Acids Res. 22:3392-3396) and the sequence of the proteolysis tag



encoded by *B. subtilis* SsrA ((A)GKTNSFNQNVALAA SEQ ID NO:1) has been predicted (Williams 2000. The tmRNA website. Nucleic Acids Res. 27:165-166). Recently, Wiegert and Schumann (2001. SsrA-mediated tagging in *Bacillus subtilis*. J. Bacteriol. 183:3885-3889) showed that the ClpXP protease is responsible for the degradation of intracellular SsrA-tagged proteins in *B. subtilis*. The instant invention provides for an enhanced protein stability via enhance protease resistance. In particular, the extracellular protease CtpA, and perhaps one or more of the major extracellular proteases of *B. subtilis*, play a role in the degradation of an extracellular, heterologous protein that was tagged by the SsrA system. It is a benefit that tagged proteins according to the present invention are more resistant to proteolysis.

#### **Possible tags that may be used**

Tags may either be added to the carboxy terminus of a protein or substituted for the amino acids of the protein's carboxy terminus. If the protein has been tagged by the addition of amino acid residues the tag is preferably up to 20 additional residues preferably about 15, more preferred 1-14, even more preferred 1-11, and most preferred 1-3, wherein the last one or two amino acid residues are charged. Figure 7D depicts a protein with a tag added on to its carboxy terminus (SEQ ID NO:28). In this depiction the tag is 14 amino acid residues long.

In the alternative, the tag may replace between 1 and 5 amino acids in the protein's carboxy terminus. In the substituted tag the amino acids are charged. In a preferred embodiment the last 5 amino acids are replaced with the tag. In another preferred embodiment the last 4 amino acids are replaced with the tag. In yet another preferred embodiment the last 3 amino acids are replaced with the tag. In a more preferred embodiment the last amino acid is replaced with the tag. In a most preferred embodiment the last 2 amino acids are replaced with the tag. Figure 7C depicts a substitution tagged protein (SEQ ID NO:27). In this depiction the final two amino acid residues of the native protein have been replaced with two charged amino acid residues.

To study the degradation of SsrA-tagged proteins in *B. subtilis*, variants of pLATIL3 were made in which h-IL3 is expressed with different short peptide tags added to the COOH-terminus of h-IL3:

Variant 1: plasmid pLATIL3-BStag; expresses the hIL3 variant hIL3-AA: hIL3 with an apolar C-terminal *B. subtilis* SsrA-tag [GKTNSFNQNVALAA] (SEQ ID NO:5)

Variant 2: plasmid pLATIL3-DDtag; expresses the hIL3 variant hIL3-DD: hIL3 with a negatively charged C-terminal tag [GKTNSFNQNVALDD] (SEQ ID NO:6), this variant differs from variant 1 (hIL3-AA) only in the last two C-terminal amino acids (two aspartic acids (DD) instead of two alanine (AA) residues)

Variant 3: plasmid pLATIL3-Ectag; expresses the hIL3 variant hIL3-ECAA: hIL3 with an apolar C-terminal *E. coli* SsrA tag [AANDENYALAA] (SEQ ID NO:3)

Transformation of competent cells was used to transfer DNA (plasmids, linear DNA) into *B. subtilis* (Harwood et al. 1990). PCR (polymerase chain reaction) was carried out with High Fidelity Platinum Taq DNA Polymerase (Life technologies) and if required PCR fragments were purified with the Qiaquick PCR purification kit (Qiagen). DNA primers were from Life technologies and DNA sequencing was performed by BaseClear (Leiden, The Netherlands). Plasmid pLATIL3TERM was obtained by PCR on pLATIL3 with the primers pLATIL3SXHfw (5' GTC GAC CTC GAG ACC CCA AGC TTG GCG TAA TC 3') (SEQ ID NO:7) and pLATIL3T3rv (5' GTC GAC CTC GAG CGG CAG AAT CTT TTT TTG ATT CTG CCG CAA AGT CGT CTG TTG AGC CTG 3') (SEQ ID NO:8). The resulting DNA fragment was purified, digested with XhoI, self-ligated, and transformed directly into *B. subtilis*. One of the plasmid clones, found to be correct by DNA sequencing, was designated pLATIL3TERM. This plasmid holds the transcription terminator of the folC gene (present in primer pLATIL3T3rv) at the 3' end of the AmyL-hIL-3 gene, just in front of an in-frame stop codon. Plasmid pLATILBStag

was obtained by a PCR on pLATIL3 with the primers pLATIL3T2FW (5' CTG CAG CTC GAG GAT ATC GTC GAC CGG CAG AAT CAA AAA AAG ATT CTG CCG ACC CCA AGC TTG GCG TAA TC 3') (SEQ ID NO:9) and pIL3BstagRV (5' CTT CTA CTC GAG TCA GGC AGC TAA TGC TAC GTT TTG GTT AAA ACT GTT AGT TTT GCC TGC GCT CAA AGT CGT CTG TTG AGC 3') (SEQ ID NO:10). The resulting PCR fragment was purified, digested with XhoI, self-ligated, and transformed into *B. subtilis*. A few clones were checked by DNA sequencing and one correct clone was selected and named pLATIL3Bstag. Plasmid pLATIL3DDtag and pLATIL3Ectag were made in the same way but instead of primer pIL3BstagRV, primer pIL3DDtagRV (5' CTT CTA CTC GAG TCA GTC GTC TAA TGC TAC GTT TTG GTT AAA ACT GTT AGT TTT GCC TGC GCT CAA AGT CGT CTG TTG AGC 3') (SEQ ID NO:11) and primer pIL3EctagRV (5' CTT CTA CTC GAG TCA AGC TGC TAA AGC GTA GTT TTC GTC GTT TGC TGC GCT CAA AGT CGT CTG TTG AGC 3') (SEQ ID NO:12) were used, respectively. To construct *B. subtilis*  $\Delta$ ssrA mutants, ssrA and its flanking regions (approximately 2.2 kb) was amplified by PCR with the primers pSsrAFW (5' CAG CTC CGT CTG AGG AAA AAG 3') (SEQ ID NO:13) and pSsrARV (5' CGA AGT GGG CGA TTT CTT CCG 3') (SEQ ID NO:14) and cloned into pCR2.1-TOPO, resulting in plasmid pTPSsrA. Plasmid pSsrASp was obtained by inserting a pDG1726-derived Sp resistance marker (Guérout-Fluery et al. 1995. Antibiotic-resistance cassettes for *Bacillus subtilis*. Gene 167:335-336) into the unique SacI site in the ssrA gene of pTPSsrA. Finally, *B. subtilis* 168  $\Delta$ ssrA and WB600  $\Delta$ ssrA were obtained by a double cross-over recombination event between the disrupted ssrA gene of pSsrASp and the chromosomal ssrA gene in *B. subtilis* 168 and WB600, respectively. SsrA<sup>DD</sup> expressing *B. subtilis* strains were made as follows: a fragment consisting of a 5' end part of ssrA including the ssrA promoter region, was amplified with the primers pSsrAHindIIIfw (5' TTC TAA AAG CTT AGT GCT TGA TTC GAA AAT CAG GCC TGT G 3') (SEQ ID NO:15) and pSsrADDintRV (5' GAG CTC GCT GCG CTT ATT AGT CGT CTA ATG CTA CGT TTT GGT TAA 3') (SEQ ID NO:16); contains the alteration of the two alanine codons in the SsrA tag sequence into codons for aspartic acid residues). In addition, an overlapping 3' end part of ssrA was amplified with the primers pSSrADDintFW (5' TTA ACC AAA ACG TAG CAT TAG ACG ACT AAT AAG CGC AGC GAG CTC 3') (SEQ ID NO:17); also containing the alteration

of the two alanine codons into codons for two aspartic acid residues) and pSsrASphIRV (5' CCT CCG TGC ATG CTT CCT CTT ATT TAT TGA CAG AAA TCT G 3') (SEQ ID NO:18). Both fragments were assembled in a fusion PCR with primers pSsrAHindIIIFW and pSsrASphIRV, and cloned in pCR2.1-TOPO, resulting in plasmid pSsrADD. The correct sequence of the fusion product in pSsrADD was confirmed by DNA sequencing. Next, a selective marker (the Tc resistance cassette derived from pDG1515; Guérout-Fluery et al. 1995. Antibiotic-resistance cassettes for *Bacillus subtilis*. Gene 167:335-336) that functions in *B. subtilis*, was cloned into the EcoRV site of pSsrADD, resulting in plasmid pSsrADDTc. Finally, *B. subtilis* 168 IssrA<sup>DD</sup> and WB600 IssrA<sup>DD</sup> were obtained by a Campbell-type integration (single cross-over) of pSsrADDTc into one of the disrupted *ssrA* regions on the chromosome of *B. subtilis* 168  $\Delta$ *ssrA* and WB600  $\Delta$ *ssrA*, respectively. These strains contain an active copy of the *ssrA*<sup>DD</sup> gene on the chromosome (under control of the native *ssrA* promoter) and a disrupted copy of wild-type *ssrA* (insertion of the Sp resistance marker), as confirmed by PCR. To construct *B. subtilis* WB600  $\Delta$ *ctpA*, WB600 was transformed with chromosomal DNA of BSE-23. In BSE-23, the *ctpA* gene is replaced by a spectinomycin resistance cassette (Edwin Lee, Genencor International Palo Alto, unpublished). WB600  $\Delta$ *yvjB* was obtained as follows: *yvjB* and its flanking regions (approximately 3.5 kb) was amplified by PCR with the primers pYvjBFW (5' AGA GTT TTA AAT CTC TCG GGA GAA ACA CAT GGA TGA CAT T 3') (SEQ ID NO:19) and pYvjBRV (5' TGT ATA TGT AAA TTT CAG ATC ATC ATA AAT ATC TGC TAT T 3') (SEQ ID NO:20) and cloned in pCR2.1-TOPO, resulting in plasmid pTPYvjB. Plasmid pTPYvjBTc was obtained by replacing an internal SmaI-AccI fragment of the *yvjB* gene in pTPYvjB with a pDG1515-derived Tc resistance marker (Guérout-Fluery et al. 1995. Antibiotic-resistance cassettes for *Bacillus subtilis*. Gene 167:335-336). Finally, *B. subtilis* WB600  $\Delta$ *yvjB* was obtained by a double cross-over recombination event between the disrupted *yvjB* gene of pTPYvjBTc and the chromosomal *yvjB* gene. To construct *B. subtilis* WB600  $\Delta$ *clpP*, the 5' end region of the *clpP* gene was amplified by PCR with the primers pClpPEcoFW (5' CTT ACC GAA TTC GTG AAG GAG GAG CAT TAT G 3') (SEQ ID NO:21) containing a EcoRI site, and pClpPBamRV (5' GCC TTT GGA TCC GGC TGC AAG CAG GAA CGC 3') (SEQ ID NO:22) containing a BamHI site. The amplified fragment was cleaved with EcoRI and

BamHI, and cloned in the corresponding sites of pMutin2 (Vagner et al. 1998. A vector for systematic gene inactivation in *Bacillus subtilis*. Microbiology 144:3097-3104), resulting in plasmid pMutClpP. *B. subtilis* WB600 lclpP was obtained by a Campbell-type integration (single cross-over) of pMutClpP into the clpP region on the chromosome. Cells of this strain are depleted for ClpP by growing them in medium without IPTG (Vagner et al. 1998).

Table 1. Plasmids and Strains

Plasmid/Strain	Properties	Reference
pLATIL3	derivative of pGB/IL-322: contains the human <i>IL-3</i> gene fused to the sequence encoding the signal peptide of <i>B. licheniformis</i> $\alpha$ -amylase ( <i>amyL-hIL-3</i> ); the <i>amyL-hIL-3</i> gene fusion is under control of the amylase promoter; 4.3 kb; Nm <sup>R</sup>	Van Leen et al. 1991. Production of human interleukin-3 using industrial microorganisms. Biotechnology 9:47-52.
pLATIL3TERM	derivative of pLATIL3; contains the transcription terminator of the <i>B. subtilis</i> <i>folC</i> gene inserted just in front of the stop codon of <i>amyL-hIL-3</i> ; 4.1 kb; Nm <sup>R</sup>	This work
pLATIL3BStag	derivative of pLATIL3; contains <i>amyL-hIL-3</i> fused at the 3' end to the sequence encoding the <i>B. subtilis</i> SsrA peptide tag (AGKTNSFNQ <sup>N</sup> VALAA SEQ ID NO:1); 4.2 kb; Nm <sup>R</sup>	This work
pLATIL3DDtag	derivative of pLATIL3; contains <i>amyL-hIL-3</i> fused at the 3' end to the sequence encoding a variant SsrA- DD-tag (AGKTNSFNQ <sup>N</sup> VALDD SEQ ID NO:2); 4.2 kb; Nm <sup>R</sup>	This work
pLATIL3Ectag	derivative of pLATIL3; contains <i>amyL-hIL-3</i> fused at the 3' end to the sequence encoding the <i>E. coli</i> SsrA peptide tag (AANDENYALAA SEQ ID NO:3); 4.2 kb; Nm <sup>R</sup>	This work
pCR2.1-TOPO	TA cloning vector for PCR products; 3.9 kb; Ap <sup>R</sup> ; Km <sup>R</sup>	Invitrogen
pTPSSrA	pCR2.1-TOPO derivative; carrying the <i>ssrA</i> gene + flanking regions; 6.1 kb; Ap <sup>R</sup> ; Km <sup>R</sup>	This work
pSsrASp	derivative of pTPSSrA for the disruption of <i>ssrA</i> ; 7.0 kb; Ap <sup>R</sup> ; Km <sup>R</sup> ; Sp <sup>R</sup>	This work
pSsrADD	pCR2.1-TOPO derivative; carrying a	This work

Plasmid/Strain	Properties	Reference
	<i>ssrA<sup>DD</sup></i> gene variant: the last two codons of the tag sequence in <i>ssrA</i> (gct gcc) encoding two alanines are changed into gac gac, encoding two aspartic acid residues; 4.6 kb; Ap <sup>R</sup> ; Km <sup>R</sup>	
pSsrADDTc	derivative of pSsrADD; carrying <i>ssrA<sup>DD</sup></i> and a Tc resistance cassette; for integration of <i>ssrA<sup>DD</sup></i> on the <i>B. subtilis</i> chromosome; 6.8 kb; Ap <sup>R</sup> ; Km <sup>R</sup> ; Tc <sup>R</sup>	This work
pTPYvjB	pCR2.1-TOPO derivative; carrying the <i>yvjB</i> gene + flanking regions; 7.4 kb; Ap <sup>R</sup> ; Km <sup>R</sup>	This work
pTPYvjBTc	derivative of pTPYvjB for the disruption of <i>yvjB</i> ; 8.9 kb; Ap <sup>R</sup> ; Km <sup>R</sup> ; Tc <sup>R</sup>	This work
pMutin2	pBR322-based integration vector for <i>B. subtilis</i> ; containing a multiple cloning site downstream of the Pspac promoter, and a promoterless 1998 <i>lacZ</i> gene preceded by the RBS of the <i>spoVG</i> gene; 8.6 kb; Ap <sup>R</sup> ; Em <sup>R</sup>	Vagner et al. 1998. A vector for systematic gene inactivation in <i>Bacillus subtilis</i> . Microbiology <b>144</b> :3097-3104.
pMutClpP	pMutin2 derivative; carrying the 5' part of the <i>B. subtilis clpP</i> gene; 8.9 kb; Ap <sup>R</sup> ; Em <sup>R</sup>	This work
<b>Strains</b>		
<i>E. coli</i>		
TOP10	<i>F mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 deoR araD139 Δ(ara-leu)7697 galU galK rpsL (Str<sup>R</sup>) endA1 nupG</i>	Invitrogen
XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [FϕproAB lac<sup>f</sup>ZDM15 Tn10 (Tet<sup>r</sup>)]</i>	Stratagene
<i>B. subtilis</i>		
168	<i>trpC2</i>	Kunst et al. 1997. The complete genome sequence of the Gram-positive bacterium <i>Bacillus subtilis</i> . Nature <b>390</b> :249-256.
168 Δ <i>ssrA</i>	<i>trpC2, ssrA</i> ; Sp <sup>R</sup>	This work
168 <i>lssrA<sup>DD</sup></i>	<i>trpC2, lssrA<sup>DD</sup></i> ; Tc <sup>R</sup> ; integration of pSsrADDTc in <i>ssrA::spec</i> in 168 Δ <i>ssrA</i>	This work
WB600	<i>trpC, nprE, aprE, epr, bpf, mpr,</i>	Wu et al. 1991.

Plasmid/Strain	Properties	Reference
	<i>nprB</i>	Engineering a <i>Bacillus subtilis</i> expression-secretion system with a strain deficient in six extracellular proteases. J. Bacteriol. <b>173</b> :4952-4958.
BSE-23	<i>ctpA</i> ; Sp <sup>R</sup>	E. Lee, unpublished
WB600 $\Delta$ <i>ctpA</i>	<i>trpC</i> , <i>nprE</i> , <i>aprE</i> , <i>epr</i> , <i>bpf</i> , <i>mpr</i> , <i>nprB</i> , <i>ctpA</i> ; Sp <sup>R</sup>	This work
WB600 $\Delta$ <i>yvjB</i>	<i>trpC</i> , <i>nprE</i> , <i>aprE</i> , <i>epr</i> , <i>bpf</i> , <i>mpr</i> , <i>nprB</i> <i>yvjB</i> ; Tc <sup>R</sup>	This work
WB600 <i>lclpP</i>	<i>trpC</i> , <i>nprE</i> , <i>aprE</i> , <i>epr</i> , <i>bpf</i> , <i>mpr</i> , <i>nprB</i> , <i>Pspac-clpP</i> ; <i>clpP-lacZ</i> ; Em <sup>R</sup>	This work
WB600 $\Delta$ <i>ssrA</i>	<i>trpC</i> , <i>nprE</i> , <i>aprE</i> , <i>epr</i> , <i>bpf</i> , <i>mpr</i> , <i>nprB</i> , <i>ssrA</i> ; Sp <sup>R</sup>	This work
WB600 <i>lssrA</i> <sup>DD</sup>	<i>trpC</i> , <i>nprE</i> , <i>aprE</i> , <i>epr</i> , <i>bpf</i> , <i>mpr</i> , <i>nprB</i> , <i>lssrA</i> <sup>DD</sup> ; Tc <sup>R</sup>	This work

RNA isolation and Northern blotting. RNA was isolated with the TRIzol method according to the protocol provided by the manufacturer (Life technologies), but with one modification: cells were incubated for 10 min at 37 °C with lysozyme (2 mg/ml) prior to lysis in TRIzol solution. Northern blotting was performed after electrophoresis of RNA through gels containing formaldehyde (Sambrook et al. 1989). To this purpose, Hybond-N+ nylon membrane from Amersham Pharmacia Biotech was used. The SsrA-specific probe was amplified by PCR with the primers SsrAFRWDP (5' ACG TTA CGG ATT CGA CAG GGA TGG 3') (SEQ ID NO:23) and SsrAREVP (5' GAG TCG AAC CCA CGT CCA GAA A 3') (SEQ ID NO:24). Labeling of the probe, hybridization and detection was performed with the ECL direct nucleic acid labeling and detection system from Amersham Pharmacia Biotech according to the manufacturer's instructions.

Western blot analysis. To obtain anti-BsSsrA-tag antibodies (antibodies that recognize proteins with a C-terminal B. subtilis SsrA-tag), synthetic peptide AGKTNSFNQNVALAA (SEQ ID NO:1) (coupled via an amino-terminal cysteine residue to KLH carrier) was injected into rabbits (Eurogentec). Serum of the final bleed of one of the rabbits was selected for affinity purification, and this purified serum was used in the Western blot procedures. Antibodies against human IL-3 were mouse monoclonals (Van Leen et al. 1991. Production of human interleukin-3 using industrial microorganisms.

Biotechnology 9:47-52). Immunoblotting and detection was performed with alkaline phosphatase-labeled conjugate and the BM Chromogenic Western Blotting kit (Roche Diagnostics) according to the instructions of the manufacturer.

**Stability of hIL-3 variants with different C-terminal tags produced by *B. subtilis*.**

To further investigate whether the *B. subtilis* SsrA tag functions as a degradation signal for secreted proteins, three variants of plasmid pLATIL3 were created. Plasmid pLATIL3BStag contains a gene variant encoding hIL-3 fused at the C-terminus to the *B. subtilis* SsrA peptide tag (AGKTNSFNQNVALAA SEQ ID NO:1), plasmid pLATIL3ECtag contains a gene variant encoding h-IL3 fused at the C-terminus to the *E. coli* SsrA tag (AANDENYALAA SEQ ID NO:3). The third plasmid pLATIL3DDtag contains a gene encoding h-IL3 fused at the C-terminus to the sequence encoding a DD-tag (AGKTNSFNQNVALDD SEQ ID NO:2). This tag is equal to the *B. subtilis* SsrA-tag (AA-tag), but instead of two alanines at the extreme C-terminus it contains two aspartic acid residues. The DD-tag was suspected to be relatively resistant to proteolytic degradation, as observed for *E. coli* (Abo et al. 2000. SsrA-mediated tagging and proteolysis of LacI and its role in the regulation of *lac* operon. EMBO J. 19:3762-3769; Roche et al. 1999). The extracellular proteins produced by cells of *B. subtilis* 168 containing pLATIL3, pLATIL3BStag, pLATIL3DDtag, or pLATIL3ECtag, were analyzed by Western blotting (Fig. 3A). The amount of the hIL-3-DDtag present in the medium was found to be roughly 5 times higher than that of wild-type hIL-3, hIL-3-AAtag or hIL-3-ECtag. Human interleukin-3 molecules produced by wild-type *B. subtilis* are relatively unstable due to proteolytic degradation, and the results represented in figure 3A suggest that addition of a C-terminal SsrA-tag does not lead to increased degradation of hIL-3 molecules. It is important to note, however, that in *E. coli* proteins tagged cotranslationally by the SsrA system are degraded more rapidly than proteins with essentially the same sequence in which the SsrA tag is DNA encoded (Gottesman et al. 1998. The ClpXP and ClpAP proteases degrade proteins with carboxy-terminal peptide tails added by the SsrA-tagging system. Genes Dev. 12:1338-1347). The results obtained with pLATIL3TERM (Fig. 2) indicate that this is also true for *B. subtilis*. Strikingly, addition of the DD-tag (with two charged, polar residues at the extreme C-terminus) leads to a higher level of



extracellular hIL-3, indicating that DD-tagged hIL-3 is less susceptible to proteolytic degradation. To explore this further, a pulse-chase assay was performed with the *B. subtilis* strain 168 (pLATIL3BStag) and 168 (pLATIL3DDtag) (Fig. 3B and 3C). The initial level (chase time = 0 min) of hIL-3-DDtag in the medium is approximately 4 times higher than that of hIL-3-AAtag. In addition, the hIL-3-AAtag variant was degraded with a half-life of < 2 min, whereas the half-life of hIL-3-DDtag was somewhat increased (approximately 5 min). The latter observation supports that DD-tagged hIL-3 is less susceptible to extracellular proteases compared to hIL-3 with an AA-tag. However, the observation that the initial level of hIL3-DDtag in the medium is considerably higher than that of hIL3-AAtag indicates that hIL3-AAtag is also subject to proteolytic degradation before the molecules reach the medium, e.g. during passage of the cell wall of *B. subtilis*.

**Detection of cotranslationally SsrA-tagged hIL-3 secreted by WB600 and by cells expressing an SsrA<sup>DD</sup> variant.** To detect SsrA-tagged h-IL3 molecules secreted by *B. subtilis* and to identify proteases that have a role in the degradation of SsrA-tagged hIL-3, two different approaches were used. First, pLATIL3TERM was expressed in WB600, a *B. subtilis* strain lacking six extracellular proteases (Wu et al. 1991. Engineering a *Bacillus subtilis* expression-secretion system with a strain deficient in six extracellular proteases. J. Bacteriol. 173:4952-4958), which may be responsible for the degradation of extracellular, SsrA-tagged hIL-3. In the medium of a culture of WB600 (pLATIL3TERM), a band was detected reacting with antibodies against hIL-3 as well as with antibodies raised against the predicted *B. subtilis* SsrA-tag (Fig. 4, lane 3 and 8). This band is absent in the medium of *B. subtilis* 168 (pLATIL3TERM) (lanes 2 and 7) and WB600  $\Delta$ ssrA (pLATIL3TERM) (lanes 4 and 9). Thus, hIL-3 molecules translated from mRNAs that lack termination codons are tagged by *B. subtilis* SsrA. The fact that these tagged molecules react with antibodies raised against the predicted *B. subtilis* SsrA peptide tag (AGKTNSFNQ<sup>N</sup>VALAA SEQ ID NO:1) indicates that this prediction, which was based on comparative sequence analysis of SsrA sequences of several bacteria (Williams 2000), was correct. In addition, it can be concluded that at least one of the major extracellular proteases of *B. subtilis* (those that are absent in WB600) plays

a role in the degradation of extracellular, SsrA-tagged h-IL3. When SsrA is absent, stalled ribosomes are released by an SsrA-independent mechanism, referred to as 'run-off translation' (Williams et al. 1999. Resuming translation on tmRNA: a unique mode of determining a reading frame. EMBO J. 18:5423-5433). The upper band in lane 4 probably represents the run-off translation product of full-length hIL-3 mRNA from pLATIL3TERM, while the bands with lower molecular weight are most likely degradation products thereof. It seems that some run-off translation product is also formed when SsrA is present (lane 3), but it cannot be excluded that this band is just an N-terminal degradation product of SsrA-tagged hIL-3.

As a second approach to detect SsrA-tagged proteins, we constructed *B. subtilis* strains that express an SsrA variant (SsrA<sup>DD</sup>), in which the final two codons of the peptide reading frame are changed to encode aspartic acid residues instead of alanines. As mentioned above, it was shown in *E. coli* that an SsrA<sup>DD</sup> variant mediates the addition of a peptide tag that does not lead to rapid degradation (Abo et al. 2000; Karzai et al. 1999. SmpB, a unique RNA-binding protein essential for the peptide-tagging activity of SsrA (tmRNA). EMBO J. 18:3793-3799). Evaluation of the antibodies that were raised against the predicted *B. subtilis* SsrA tag (AGKTNSFNQNVALAA SEQ ID NO:1) showed that they recognize the hIL-3 fused at the C-terminus to either the wild-type tag (AA-tag) or the protease resistant DD-tag (AGKTNSFNQNVALDD SEQ ID NO:2) (data not shown). Human IL-3 molecules tagged by SsrA<sup>DD</sup> and subsequently secreted (Fig 4, lanes 5 and 10) are indeed relatively more stable than hIL-3 molecules tagged by wild-type SsrA (lanes 3 and 8), even in the six-fold protease negative strain WB600. The level of full-length SsrA<sup>DD</sup>-tagged hIL-3 in the medium is somewhat higher than that of (wild-type) SsrA-tagged h-IL3 (Fig. 3, compare lane 10 with lane 8) and relatively few degradation products of SsrA<sup>DD</sup>-tagged hIL-3 were detected with anti-hIL-3 antibody (compare lane 5 with lane 3). This observation suggests that besides the major extracellular proteases that are deleted in WB600, one (or more) additional protease is involved in the degradation of SsrA-tagged hIL-3. Therefore, we studied the role of three other proteases with respect to degradation of SsrA-tagged hIL-3.

In the Claims:

37. The recombinant protein of interest of Claim 34 wherein said positively charged residue is R [N].

38. The recombinant protein of interest of Claim 29 wherein said tag is selected from the group comprising SsrA<sup>NNRR</sup> (SEQ ID NO:30), SsrA<sup>DD</sup> (SEQ ID NO:2), SsrA<sup>KK</sup> (SEQ ID NO:4), and SsrA<sup>EE</sup> (SEQ ID NO:29).

48. The nucleic acid molecule of claim 46 wherein the charged amino acid residue is R [N].